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3. Chien, J. et al. Oncogene (1999) 18(22): 3376-3382
4. Wong, E.C.C. et al. Proc. Amer. Assoc. for Cancer Res. (1997) 38: 288
5. Rayford, W. et al. Prostate (1997) 30(3): 160-166
6. Xue-Zhang, Q. et al. Endocrine (1995) 3(6): 445-451
7. Shah, G.V. et al. Endocrinology (1994) 134(2): 596-602
8. Rayford, W. et al. J. of Urology (1994) 151(5 suppl): 490A
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10. Shah, G.V. et al. Prostate (N.Y.) (1992) 21(2): 87-97
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12. Sussenot, O. et al. Prostate (1998) 36(suppl. 8): 43-51
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Calcitonin inhibits prolactin gene transcription in rat pituitary cells

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Our recent studies have shown that calcitonin (CT)-like immunoreactive peptide is synthesized and released from cultured rat anterior pituitary (AP) cells, and may serve as a paracrine inhibitor of PRL release. The present studies investigated effects of CT on basal and TRH-induced PRL mRNA levels in rat AP and rat pituitary tumor GH₃ cells. CT attenuated steady-state PRL mRNA levels in a dose-dependent fashion in primary rat AP and GH₃ cells. The kinetics of CT action suggests that 100 nM CT caused a significant decline after 3 h, and the inhibition was sustained at least until the longest tested incubation period of 30 h. Results from nuclear run-on assays suggest that 100 nM CT decreased the rate of PRL gene transcription by 80% after 30 min of incubation. CT did not affect PRL mRNA levels in Ca²⁺-depleted GH₃ cells but dramatically decreased them in Ca²⁺-repleted cells. Bay K 8644 induced increase in PRL mRNA levels of Ca²⁺-repleted GH₃ cells and CT did not affect this increase. These results suggest that CT rapidly and selectively inhibits PRL gene transcription in primary AP and GH₃ cells, and support a possibility that CT-induced attenuation of PRL mRNA may involve cytoplasmic Ca²⁺.

Keywords: calcitonin; paracrine inhibition; prolactin; gene transcription; pituitary gland

Introduction

Expression of PRL gene is regulated by various hypothalamic and peripheral hormones (Maurer, 1981, 1982; Potter *et al.*, 1981; Abe *et al.*, 1985; Lamberts *et al.*, 1989; Lamberts & MacLeod, 1990). Recent studies have shown that subpopulations of pituitary lactotrophs exhibit heterogeneity as assessed by their basal and stimulated PRL gene transcription (Bockfor & Frawley, 1987; Velkeniers *et al.*, 1988; Arita *et al.*, 1992; Kazemzadeh *et al.*, 1992). This raises a possibility that PRL biosynthesis within lactotroph subpopulations may be modulated by locally secreted paracrine/autocrine factors. Several peptidergic factors that directly affect PRL gene expression have been shown to be synthesized and released by cultured AP cells (Kudlow & Kobrin, 1984; Arnaout *et al.*, 1986; Deschepper *et al.*, 1986; Kaplan *et al.*, 1988). Recent findings from this and other laboratories have shown that exogenously added CT selectively and potently inhibits basal and TRH-stimulated PRL release from rat AP cells (Shah *et al.*, 1988; Judd *et al.*, 1990). CT does not affect basal GH, TSH, FSH and LH release as well as TRH-induced TSH or GnRH-induced LH release (Shah *et al.*, 1990b). These studies have further shown that immunoreactive calcitonin (CT)-like peptide is synthesized and released from cultured rat AP cells and rabbit anti-CT serum induces a significant increase in basal PRL release from cultured rat AP cells (Shah *et al.*, 1992). These results suggest that pituitary-derived CT-like peptide may serve as a paracrine/autocrine inhibitor of PRL release. An important objective of the present investigations was to

examine whether CT affects steady-state and TRH-induced PRL gene expression.

GH₃ is a well-characterized rat pituitary tumor-derived cell line that has been shown to retain several properties of lactotrophs (Tashjian, 1979; Bancroft, 1981). For example, GH₃ cells respond to estrogens, corticosteroids as well as TRH and have been widely used to study mechanisms associated with PRL gene expression. The present study tested effects of CT on PRL mRNA levels in primary rat AP and GH₃ cells. Additional experiments examined effects of CT on PRL gene transcription as well as on TRH-induced PRL mRNA levels. Since previous findings suggest that CT also inhibits TRH-induced inositol phosphates generation (Judd *et al.*, 1990), a possibility that cytoplasmic Ca²⁺ mediates the actions of CT on PRL gene expression was tested.

Results

Effect of CT on steady-state PRL mRNA levels in primary AP cells and GH₃ cells

We began this study by testing the effects of various concentrations of sCT on PRL mRNA levels in primary rat AP cells. RNA samples from these experiments were dot-blotted to accommodate several replicates at various concentration points. The results presented in top panel of Figure 1 have shown sCT attenuated steady-state PRL mRNA levels in a dose-dependent fashion. The blots were then stripped off [³²P]PRL cDNA probe and reprobed with ³²P-labeled β-actin mRNA (lower panel of Figure 1). These results suggest that CT induced a marked decline in PRL mRNA levels in dose-dependent fashion, but did not appreciably alter β-actin mRNA levels. Autoradiograms from four similar, but independent, experiments were analysed by laser densitometry. Relative PRL mRNA abundance was calculated by obtaining the ratio of PRL mRNA levels to β-actin mRNA levels from same dots. Analysis of pooled data from these experiments suggests that sCT significantly attenuated PRL mRNA levels at concentrations of 10 nM and higher. 100 nM sCT induced a maximal (61%) decline in PRL mRNA concentrations (Figure 1).

In a second group of experiments, effect of various sCT concentrations on PRL mRNA levels in GH₃ cells was examined. sCT again induced a dose-dependent decrease in PRL mRNA concentrations of GH₃ cells (Figure 2). The attenuation of PRL mRNA levels by CT was significant at all concentrations tested (0.1 nM–1 μM), and a maximal decline of 60% was observed at 100 nM concentration. Again, the inhibitory action of sCT was selective for PRL mRNA expression and did not affect β-actin mRNA levels (Figure 2). Because of similarity of responses by primary AP and GH₃ cells, subsequent experiments used GH₃ cells as model to characterize inhibitory actions of CT on PRL gene expression. Moreover, GH₃ cells offer several advantages over primary AP cells. For example, they are homogeneous and can be grown in culture in unlimited amounts.

The results presented in Figure 3 show the time course of sCT effect in GH₃ cells. Northern analysis of RNAs from these experiments suggest that PRL mRNA levels declined significantly as early as 3 h after sCT treatment. A maximal

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Received 26 January 1995; accepted 7 March 1995

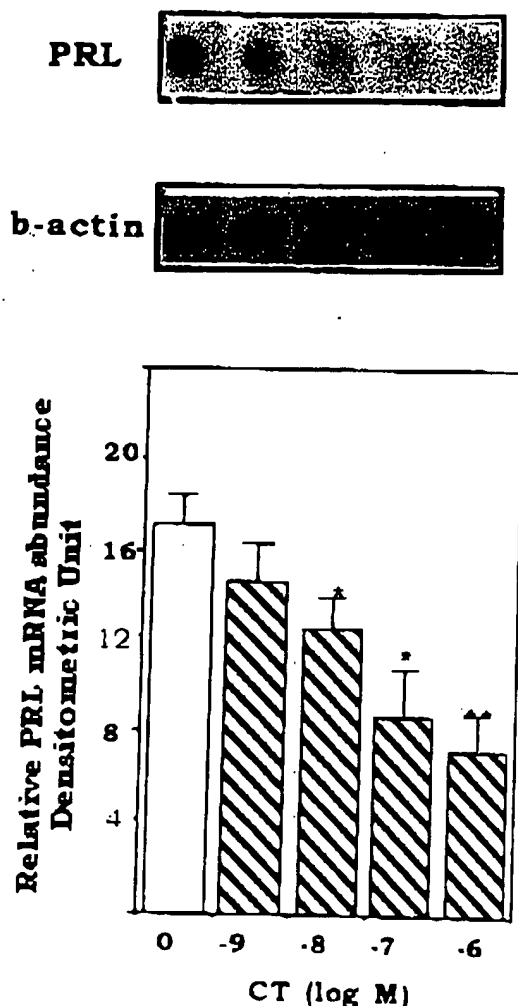


Figure 1 CT attenuates steady-state PRL mRNA levels in rat AP cells. Rat AP cells were incubated with various concentrations of CT (1 nM – $1 \mu\text{M}$) for 16 hours. Total cellular RNA was extracted and concentrations of PRL mRNA levels were analysed by sequential dot-blot hybridizations with ^{32}P -labeled PRL and ^{32}P - β -actin cDNA probes. A representative autoradiogram (of four independent experiments) showing PRL mRNA (upper panel) and β -actin mRNA signals (lower panel). The hybridization signals were quantitated by laser scan densitometry. Relative PRL mRNA abundance is calculated as ratio of PRL mRNA to β -actin mRNA ($\times 100$). The results are mean \pm SEM for $n=4$. Level of significance (between control and treated): * $P<0.01$; (between control and treated): ** $P<0.005$; (One way ANOVA and Newman-keuls test)

suppression of 72% was observed at 3 h. During longer incubation periods of 6, 21 and 30 h, a gradual increase in PRL mRNA levels was observed, although, the levels were significantly lower than the control levels at all time points. This small recovery in PRL mRNA synthesis may have been caused by inactivation or proteolysis of the CT during longer periods of incubation. Similar results were obtained in at least four similar but separate experiments. Again, sCT did not affect β -actin mRNA levels in these samples.

Effect of CT on PRL gene transcription

The rapidity of sCT response clearly suggested a transcriptional effect, which we sought to confirm by nuclear run-on assay. Initial experiments characterized experimental conditions for nuclear run-on assays and tested specificity of the hybridization signal. Figure 4 demonstrates that hybridization signal from PRL transcripts can be displaced by excess sense PRL mRNA. In these experiments, run-on RNA trans-

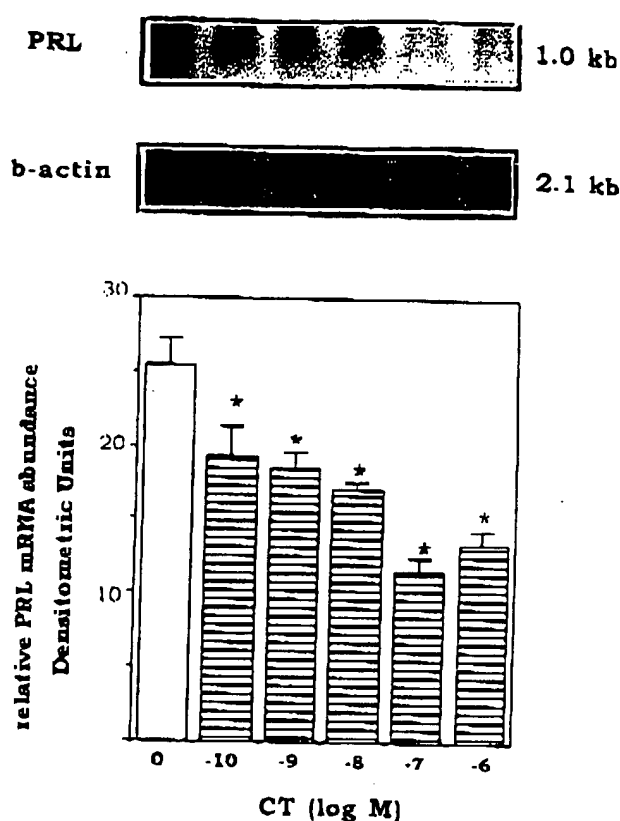


Figure 2 Attenuation of steady-state PRL mRNA levels by CT in GH₃ cells: Northern analysis. GH₃ cells were incubated with various concentrations of CT (0.1 nM – $1 \mu\text{M}$) for 16 h. Total cellular RNA was extracted and concentrations of PRL mRNA levels were analysed by Northern hybridizations. The blots were stripped off the bound PRL cDNA probe and reprobed with ^{32}P - β -actin cDNA. A representative autoradiogram (of four independent experiments) showing PRL mRNA (upper panel) and β -actin mRNA hybridizations (lower panel). The autoradiograms were scanned by laser densitometry. The results are presented as mean relative PRL mRNA levels (ratio of PRL mRNA to β -actin mRNA $\times 100$) \pm SEM for $n=4$. Level of significance (between control and treated): * $P<0.01$ (One way ANOVA and Newman-keuls test)

cripts were hybridized with PRL cDNA strips in the absence and presence of excess sense PRL mRNA. In a second experiment, extent of RNA polymerase II-independent PRL gene transcription was examined. In these experiments, elongation of primary transcripts was performed in the absence and presence of α -amanitin ($2 \mu\text{g/ml}$). The results presented in Figure 5 show a barely detectable presence of PRL and β -actin mRNA transcripts when elongated in the presence of α -amanitin. Since α -amanitin is a specific inhibitor of RNA polymerase II, it is likely that most of PRL and β -actin gene transcription in our assays is RNA polymerase II-dependent.

We next examined effects of CT on the rate of PRL gene transcription. Initially, we performed nuclear run-on analysis at various time points upto 1 h after sCT addition, and observed that the maximal inhibition was achieved after 30 min of incubation and no further attenuation was seen at 60 min. On the basis of these results, we next performed several experiments where we examined effects of CT after 0, 15 and 30 min of incubation (a representative is presented in Figure 6). Transcriptional response to sCT was rapid as indicated by a significant 46% inhibition of PRL gene transcription within 15 min of the peptide addition and a higher inhibition of 80% was observed at 30 min. Specificity of the hybridization was further demonstrated by a complete lack of hybridization with the carrier plasmid (pSV2).

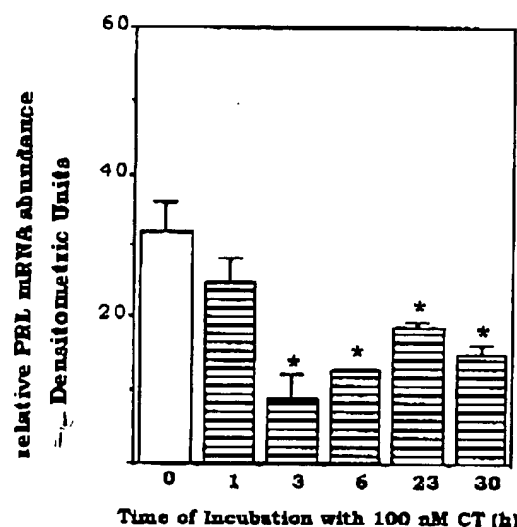
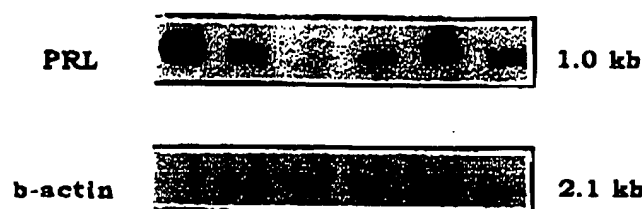


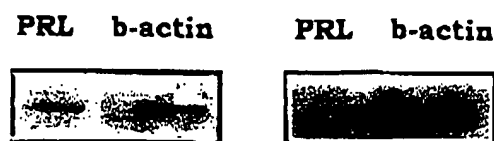
Figure 3 Time course of sCT action on PRL mRNA levels in GH₃ cells. GH₃ cells were incubated with 100 nM sCT for various time periods. Total cellular RNA (20 µg) was subjected to Northern blot analysis and probed sequentially with ³²P-labelled PRL and β-actin cDNAs. A representative autoradiogram depicting PRL mRNA signals after 2-day exposure (upper panel), and β-actin mRNA signal after 1-day exposure (lower panel). The autoradiograms were scanned by laser densitometry and absorbances for the bands were calculated. Relative PRL mRNA levels are presented as ratio of PRL mRNA to β-actin mRNA × 100. The data were analysed by one way ANOVA and the significance was derived by Newman-keuls test. **P* < 0.05

Effect of CT on TRH-induced PRL mRNA levels

Although TRH induces transcription of PRL gene acutely, the resultant increase in accumulated PRL mRNA takes a longer time. It has been reported that TRH (300 nM) takes almost 6 h to cause a twofold increase in PRL mRNA levels in GH₃ cells (Murdoch *et al.*, 1983). Therefore, incubations in this series of experiments were conducted for a longer incubation of 6 h, however, a higher concentration of 1 µM TRH was used. After 6 h of incubation, 1 µM TRH caused a large increase in PRL mRNA levels (Lane 2 of top inset in Figure 7). 100 nM CT on the other hand caused a marked decline in PRL mRNA levels as compared to vehicle-treated GH₃ cells (Lane 4 of top inset). When the cells were incubated with both the peptide hormones, sCT almost abolished TRH-induced increase in PRL mRNA levels (Lane 3 of top inset in Figure 7). In all these conditions, no appreciable changes in β-actin mRNA levels were observed (lower inset in Figure 7). Densitometric analysis of the pooled data suggest that 1 µM TRH induced a threefold increase in PRL mRNA levels. These results are similar to previously reported findings of Murdoch *et al.* (1983). CT induced a 67% decline, and combined treatment of CT and TRH almost abolished TRH-induced increase in PRL mRNA levels (Figure 7).

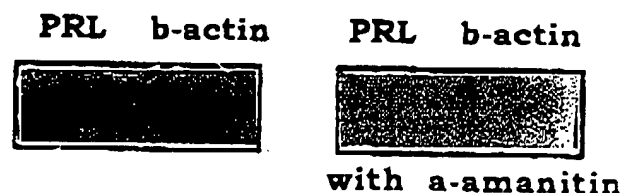
Ca²⁺ and sCT action on PRL mRNA levels

Since sCT attenuates TRH-induced increases in cytoplasmic Ca²⁺ levels (Shah *et al.*, 1990), the present study examined



with excess sense PRL mRNA

Figure 4 Characterization of nuclear run-on assay: displacement of nuclear PRL mRNA with sense PRL mRNA. Primary transcripts from GH₃ cell nuclei were labeled with [³²P]UTP in a nuclear run-on assay. The labeled nuclear RNAs were then extracted and hybridized in the presence and absence of excess sense PRL mRNA (1 µg). Left panel shows PRL mRNA hybridization signal obtained in the absence of exogenously added sense PRL mRNA. Right panel shows the PRL mRNA signal in the presence of 1 µg sense PRL mRNA. The presence of sense PRL mRNA during hybridization caused an almost complete extinction of PRL mRNA signal. β-actin mRNA signal was unaffected by the presence of sense PRL mRNA



with α-amanitin

Figure 5 Characterization of nuclear run-on assay: effect of α-amanitin on PRL gene expression. To estimate the extent of RNA polymerase II-independent gene transcription, GH₃ cell nuclei were incubated with/without α-amanitin (2 µg/ml) during elongation of primary transcripts. Nuclear RNAs were then extracted and hybridized with Nytran strips containing PRL and β-actin cDNA inserts. A typical autoradiogram presents the hybridization signals under both conditions. α-amanitin caused a dramatic decline in incorporated radioactivity (by 80–85%). A similar decline was also observed in PRL and β-actin transcripts

the role of Ca²⁺ in sCT-induced suppression of PRL mRNA levels. The results presented in Figure 8(A and B) show the effects of CT on PRL mRNA levels in Ca²⁺-depleted and Ca²⁺-repleted GH₃ cells (Lanes 1 and 2 of Figures 8A and B respectively). The results indicate that CT attenuated PRL mRNA levels of Ca²⁺-repleted cells but did not affect PRL mRNA levels in Ca²⁺-depleted GH₃ cells. To test whether CT attenuates PRL mRNA levels induced by the influx of extracellular Ca²⁺. We examined effects of CT on Bay K 8644-induced PRL mRNA levels. It has been shown that Bay K 8644 activates voltage-gated channels to increase the influx of extracellular Ca²⁺ and also induces an increase in PRL mRNA levels (28–30). The results presented in lane 3 of Figure 8A indicate that Bay K 8644 did not affect PRL mRNA levels in the absence of extracellular Ca²⁺, but significantly increased PRL mRNA levels in Ca²⁺-repleted GH₃ cells (Lane 3 of Figure 8B). Furthermore, CT did not significantly affect Bay K 8644-induced PRL mRNA expression (Lane 4 of Figure 8B).

Discussion

Expression of prolactin gene is regulated by various hypothalamic and peripheral hormones such as TRH, DA, estrogens and corticosteroids (Potter *et al.*, 1981; Maurer, 1981; Abe *et al.*, 1985; Velkeniers *et al.*, 1988; Lamberts *et al.*, 1989; Lamberts & MacLeod, 1990). However, recent evidence suggests that one more layer of regulation may also exist at the level of pituitary gland. For example, subpopulations of lactotrophs exhibit heterogeneity with respect to PRL mRNA content and their responsiveness to DA and

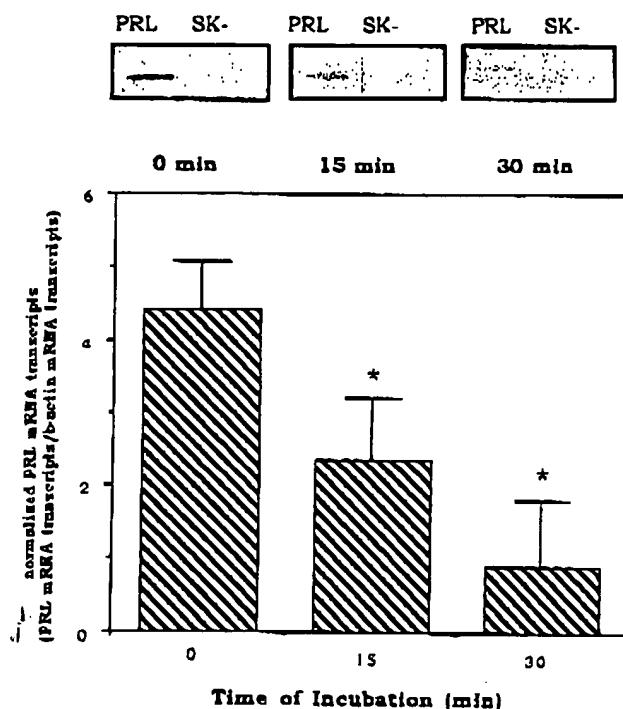


Figure 6 Inhibition of PRL gene transcription by sCT. GH₃ cells were treated with/without 100 nM sCT for 0–30 min. The cells were harvested and nuclear run-on assays were performed. Equal amounts of run-on RNAs (as assessed by incorporated radioactivity or cpm) were hybridized to cDNA strips carrying excised PRL cDNA insert and linearized pBSK. Typical autoradiograms after 5-day exposure show the concentrations of PRL transcripts at various time points (Top panels). The autoradiograms from these experiments were individually scanned by laser densitometry. Pooled results (mean \pm SEM) from three independent experiments are presented as arbitrary densitometric units. The data were analysed by *t*-test and the significance was derived from a two-tailed table. * $P < 0.01$

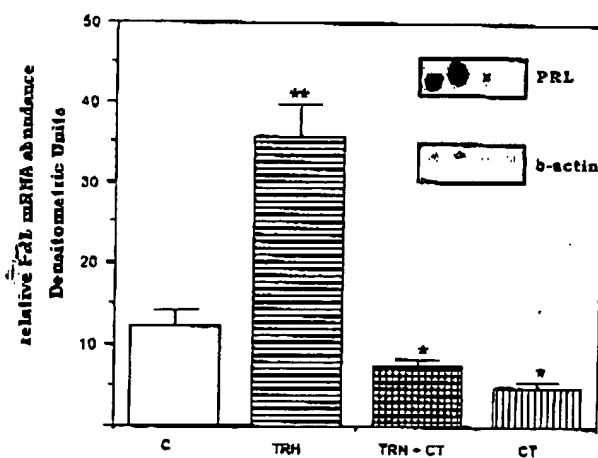


Figure 7 Effect of CT on TRH-induced PRL mRNA. GH₃ cells were incubated with vehicle (5 μ l distilled water), 1 μ M TRH, 100 nM sCT or TRH (1 μ M) + sCT (100 nM). The cells were lysed after 6 h of incubation with the hormones, cellular RNA was extracted, and Northern analysis was performed with 20 μ g of total RNA per lane. The blots were sequentially probed with ³²P-labeled PRL and β-actin cDNA probes. A typical autoradiogram depicting PRL (top inset) and β-actin mRNA signals (bottom inset). The autoradiograms from three independent experiments were individually scanned by laser densitometry, and the results are presented as mean relative PRL mRNA abundance (ratio of PRL mRNA to β-actin mRNA \times 100) \pm SEM for $n = 3$. Left to right, the RNA slots indicate control, CT, TRH and CT + TRH. The data were statistically evaluated by *t*-test, and the level of significance was derived from a two-tailed table. * $P < 0.01$; ** $P < 0.005$

TRH (Bockfor & Frawley, 1987; Velkeniers *et al.*, 1988; Arita *et al.*, 1992). Secondly, various peptides that directly affect PRL gene expression are also secreted by the AP gland (Kudlow & Kobrin, 1984; Arnaout *et al.*, 1986; Descheppe *et al.*, 1986; Kaplan *et al.*, 1988). Previous results from this laboratory have demonstrated that pituitary-derived CT-like immunoreactive peptide may serve as a paracrine inhibitor of PRL release (Shah *et al.*, 1992). Present results provide a new evidence that sCT, which may serve as an agonist to the endogenous pit-CT, attenuates PRL mRNA expression in rat AP cells. The results have shown that primary rat AP cells as well as GH₃ cells respond to sCT by showing a marked, dose-dependent decrease in steady-state PRL mRNA levels. The kinetics of CT action suggests that inhibitory effect of CT on PRL mRNA expression became observable within 1 h and a maximal decline was seen after 3 h. These rapid changes in PRL mRNA levels could most likely occur at the level of transcription. The results from nuclear run-on assays confirm this possibility and demonstrate that CT caused a rapid and dramatic decline in the rate of PRL gene transcription within 30 min and this was comparable in magnitude to its effect on steady-state PRL mRNA levels. When considered together, these results suggest that sCT affects the transcription of PRL gene rapidly, but a comparable decline in PRL mRNA occurs only after 3 h. This may be due to a longer half-life of PRL mRNA molecules which has been reported to be under 2 h in GH₃ cells (Preston *et al.*, 1990). Thus, CT, like other neuroendocrine regulators such as TRH and DA, regulates the expression of PRL gene at the level of transcription (Elsholz *et al.*, 1991; Yan & Bancroft, 1991).

Previous studies from this and other laboratories have shown that CT attenuates TRH response by inhibiting inositol phosphates generation and subsequent increases in cytoplasmic Ca²⁺ concentrations (Judd *et al.*, 1990; Shah *et al.*, 1990a). Since CT also attenuates TRH-induced PRL mRNA levels, it is likely that the action of CT on PRL gene expression is mediated by Ca²⁺/phospholipid signaling mechanisms. There is evidence to suggest that alterations in cytoplasmic Ca²⁺ concentrations have been shown to modulate PRL gene expression (Bandopadhyay & Bancroft, 1989; Day & Maurer, 1990; Enyeart *et al.*, 1990; Preston *et al.*, 1990; Elsholz *et al.*, 1991; Yan & Bancroft, 1991; Delidow *et al.*, 1992). For example, depletion of intracellular Ca²⁺ leads to decrease in PRL mRNA levels, and repletion of Ca²⁺ in Ca²⁺-depleted GH₃ cells induces a large increase in PRL mRNA levels (Bandopadhyay & Bancroft, 1989; Delidow *et al.*, 1992). By applying this previously characterized model, present study tested the effect of CT on PRL mRNA levels in Ca²⁺-depleted and Ca²⁺-repleted GH₃ cells. The results have shown that CT did not affect PRL mRNA levels in the absence of Ca²⁺ (in Ca²⁺-depleted GH₃ cells). However, it induced a dramatic decline in GH₃ cells that were repleted with Ca²⁺. These results suggest that CT attenuates Ca²⁺-inducible PRL gene expression or the presence of Ca²⁺ is required for the action of CT on PRL gene expression. To examine a possibility that CT attenuates PRL mRNA expression induced by the influx of extracellular Ca²⁺, additional studies tested the effect of CT on Bay K 8644-induced PRL mRNA expression. Bay K 8644 is a Ca²⁺ channel agonist and increases the influx of extracellular Ca²⁺ by activating voltage-gated Ca²⁺ channels (Day & Maurer, 1990; Enyeart *et al.*, 1990; Delidow *et al.*, 1992). Bay K 8644 has also been shown to increase PRL mRNA levels in GH₃ cells (Day & Maurer, 1990; Enyeart *et al.*, 1990; Delidow *et al.*, 1992). In consistence with this evidence, present results have also shown that Bay K 8644 induced a significant increase in PRL mRNA levels in Ca²⁺-repleted GH₃ cells. However, it had no effect on PRL mRNA levels in the absence of Ca²⁺, and further confirm that stimulatory action of Bay K 8644 on PRL mRNA levels is Ca²⁺-dependent. Since CT did not attenuate Bay K 8644-induced PRL mRNA expression, it is likely that CT regulates PRL gene expression by affecting the mechanisms upstream of Bay K 8644-

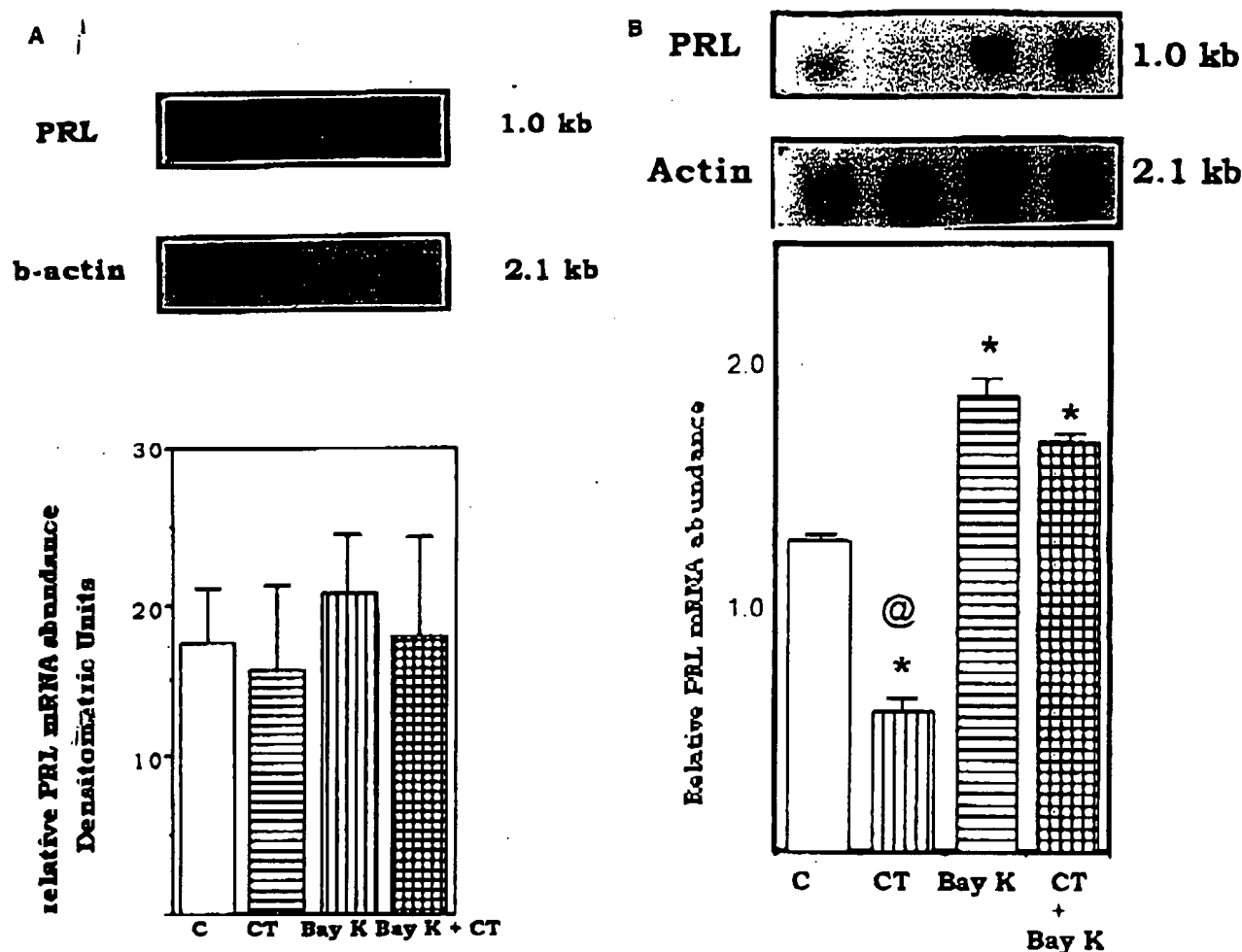


Figure 8 Effect of CT on Ca^{2+} -induced PRL mRNA levels in Ca^{2+} -deprived GH₃ cells. (A) GH₃ cells were preincubated in SFM (Ca^{2+} -free, serum-free chemically-defined medium) for 2 days and then treated with agonists as described in the Methods section. Total cellular RNA (20 μg) was subjected to Northern blot analysis and probed sequentially with ^{32}P -labelled PRL and β -actin cDNA probes. A typical autoradiogram presents the PRL mRNA signals in top panel (4-day exposure); and β -actin mRNA signal in lower panel (2-day exposure). Autoradiograms from four independent experiments were scanned by laser densitometry. Relative PRL mRNA levels are presented as arbitrary densitometric units. The data were analysed by *t*-test. * $P < 0.01$ (significantly different from control). (B) GH₃ cells were preincubated in SFM (Ca^{2+} -free, serum-free chemically-defined medium) for 2 days and then incubated with 0.5 mM CaCl_2 and other agonists for 6 h. Total cellular RNA (20 μg) was subjected to Northern blot analysis and probed with ^{32}P -labelled PRL cDNA. Top panel presents a typical autoradiogram depicting PRL mRNA signal (3-day exposure). β -actin cDNA in the same blot is presented in the lower panel (2-day exposure). Autoradiograms from four independent experiments were scanned by laser densitometry. Mean relative PRL mRNA levels \pm SEM are presented as arbitrary densitometric units. The data were analysed by *t*-test. * $P < 0.01$ (significantly different from control); @ $P < 0.03$ (significantly different from Bay K).

induced increases in cytoplasmic Ca^{2+} concentrations. Additional studies will be necessary to identify the intracellular mechanisms associated with CT-induced attenuation of PRL gene transcription.

Physiological significance of CT in regulation of PRL gene expression remains to be examined. Evidence from this and other laboratories has shown that sCT is a potent and selective inhibitor of PRL release in rats and humans (Isaac *et al.*, 1980; Pun *et al.*, 1987; Judd *et al.*, 1990; Shah *et al.*, 1990b). sCT-like immunoreactive peptide has been detected in various regions of human and rat brain, pituitary, hypothalamus, as well as various body fluids (Fischer *et al.*, 1983; Groppe *et al.*, 1985). Although the precise sequence of sCT-like mammalian peptide remains to be determined, the peptide immunoreacts with antiserum raised against sCT, co-elutes with synthetic sCT on reverse phase high performance liquid chromatography, and mRNA preparations from human thyroid glands direct the synthesis of anti-sCT immunoprecipitable peptide (Fischer *et al.*, 1983; Lasmoles *et al.*, 1985; Shah *et al.*, 1989). Receptors recognizing sCT, but not rat CT, have been detected in specific regions of rat brain and the AP gland, and cDNAs for two such receptors have recently been cloned from rat brain cDNA library (Albrandt *et al.*, 1993; Henke *et al.*, 1983; Maurer *et al.*, 1983). Therefore, it is conceivable that pit-CT, which shares antigenic epitopes with sCT and hCT, may serve as an endogenous ligand for sCT receptors in the AP gland. Our recent results that CT-like immunopositive cells are more abundant in the inner zone of rat AP gland further suggests the role for the peptide in location-dependent functional heterogeneity of lactotrophs (in preparation). Thus, CT, in concert with other paracrine and hormonal factors, may play an important role in regulating PRL gene expression; and modulating responsiveness of lactotrophs to neurohormones during different physiological conditions. In summary, the present results have shown that CT exerts potent and rapid suppression in PRL gene transcription and this effect seems to be mediated through cytoplasmic Ca^{2+} .

induced increases in cytoplasmic Ca^{2+} concentrations. Additional studies will be necessary to identify the intracellular mechanisms associated with CT-induced attenuation of PRL gene transcription.



Materials and methods

Materials

Synthetic peptides salmon (s) CT and TRH were obtained from Peninsula Laboratories, Belmont, CA. Guanidinium thiocyanate was purchased from Amresco Inc (Solon, OH). The random primed DNA-labeling kit and restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN) or US Biochemical Corporation (Cleveland, OH). [α - 32 P]Deoxy-cytidine triphosphate (dCTP; 3000 Ci/mmol) and [32 P]uridine triphosphate (UTP; 3000 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA solution, RPMI-1640 medium, Penicillin G-Streptomycin mixture, horse and fetal calf sera were obtained from Gibco Laboratories (Grand Island, NY). All other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO).

DNA clones

cDNA clones for rat prolactin (pPRL-I) and β -actin were kindly provided by Drs R Maurer (University of Iowa College of Medicine, Iowa City) and J. Calvet (University of Kansas Medical Center, Kansas City) respectively.

Animals Adult Holtzman female rats (60–90 days old) from Harlan Inc. (Milwaukee, WI) were housed two to a cage. The animals were maintained under conditions of 12 h of light and 12 h of darkness (lights on at 0600 h), with *ad libitum* access to Purina rat chow (Ralston Purina, St. Louis, MO) and tap water. After allowing for a 4-day period of acclimatization, the rats were sacrificed on random days of cycles to obtain the pituitary glands. Euthanasia was performed by decapitation under ketamine anesthesia. Protocols for the surgery as well as euthanasia have been approved the Animal Care Committee at the University of Kansas Medical Center.

Cell culture

Rat AP glands were minced and enzymatically dispersed to obtain a single cell suspension as previously described (Shah *et al.*, 1988). Dispersed AP cells were plated in six-well culture plates at a density of 4×10^6 cells per well and cultured in 5 ml of cell culture medium (RPMI medium supplemented with 15% horse serum, 5% fetal calf serum, 100 units per ml penicillin and 100 μ g per ml streptomycin) for 4 days at 37°C in a humidified 95% air and 5% CO₂ atmosphere. On fourth day of the culture, AP cells were washed and incubated with peptide hormones in a serum-free basal medium (DMEM containing 0.3% BSA, 10 mM HEPES, 2.8 mg/100 ml bacitracin and antibiotics) for 16 h. These cells were then washed in ice-cold phosphate-buffered saline (PBS) and processed for RNA extractions.

GH₃ cells GH₃ cells were obtained from the American Type Culture Collection (ATCC No. CCL 82.1, Rockville, MD) and propagated in several T 75 flasks (Falcon Plastics, Oxnard, CA) in cell culture medium (DMEM containing 5% fetal calf serum, 15% horse serum and other additives described above) under standard culture conditions. The cells from confluent flasks were harvested, seeded in 100 mm tissue culture dishes and cultured for 6 additional days with a medium change after 3 days. The cells were usually confluent at this stage. The cells were then washed with basal DMEM and incubated with various concentrations of peptide hormones. At the end of incubation, the cells were washed in ice-cold PBS and processed for RNA extractions or transcriptional analysis.

Ca²⁺ and PRL mRNA expression

The culture conditions for these experiments were modified as previously described (White & Bancroft, 1987). In brief,

confluent GH₃ cells (grown in 100 mm culture dishes) were incubated in serum-free, chemically defined Ca²⁺-free Joklik's medium (SFM) for 48 h. SFM was replaced either with Ca²⁺-free or Ca²⁺-repleted SFM (containing 0.5 mM CaCl₂), and the cells were treated with 100 nM CT and/or 300 nM Bay K 8644 for 6 h. The cells were then lysed, and processed for RNA extraction and Northern Analysis.

RNA preparation, Northern and nuclear run-on transcription assay

Cellular RNA from either GH₃ or primary AP cells was extracted by the modified method of Chomczynski and Sacchi (1987) as described by Xie and Rothblum (1991). In brief, the cells were lysed directly on culture dishes using a single-step acid-guanidinium thiocyanate-phenol-chloroform extraction. RNAs were precipitated in isopropanol, treated with proteinase K, reextracted in phenol: chloroform (1:1) and stored as precipitates at -20°C until ready for use. Total RNA (15–20 μ g/well) was fractionated on 1% agarose-formaldehyde gel and transferred to a Nytran membrane by capillary transfer (Davis *et al.*, 1986). RNA samples from primary AP cells were dot-blotted (2 μ g/well and lower) on Nytran membranes (Davis *et al.*, 1986).

cDNA probes for pPRL and β -actin were generated by excising cDNA inserts from their respective plasmids. The inserts were separated on 1% low melting point agarose gel, extracted, reprecipitated and labeled with [α - 32 P]dCTP by random primer labeling employing a kit obtained from US biochemical Corp (Cleveland, OH). Denatured DNA probes (approximately 1×10^6 cpm per ml) were hybridized with prehybridized Nytran membranes containing RNA samples. Hybridization reactions with RNA blots were continued for 18 h at 55°C. Blots were then removed, and washed with high stringency (0.1 \times SSC and 0.5% SDS) at 65°C, air-dried and autoradiographed.

Nuclear run-on transcription assays

Incubation of GH₃ cells and preparation of nuclei Confluent GH₃ cells (grown in 100 mm culture dishes) were washed in basal medium and incubated with 100 nM sCT for various incubation periods. At the end of incubation, the cells were suspended in ice and washed twice with ice-cold PBS. The cells were then treated with ice-cold RNase-free lysis buffer (Tris-HCl, pH 8.0, 3 mM CaCl₂, 2 mM MgCl₂, 0.5 mM DTT, 0.3 M sucrose, 0.15% triton X100) to isolate cell nuclei by the method of Blum (1989).

Nuclear run-on assay

Nuclei from three replicate 100 mm GH₃ dishes (approximately 15×10^6 nuclei per data point) were pooled and suspended in nuclei suspension buffer (20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF and 50% glycerol). Suspended nuclei were then added to an equal volume of 2 \times transcription mixture [containing the final concentration of 100 mM Tris-HCl, pH 7.9, 4 mM MgCl₂, 2 mM MnCl₂, 1.2 mM DTT, 0.1 mM PMSF, 0.4 mM EDTA, 10 mM creatine kinase, 0.6 mM ATP, 0.6 mM CTP and 300 μ Ci of [α - 32 P]UTP (>3000 Ci/mmol) in a total volume of 200 μ l]. Primary transcripts were allowed to elongate in a run-on transcription assay for 15 min at 20°C. A parallel experiment examined RNA polymerase II-independent transcription under identical conditions, but in the presence of α -amanitin (2 μ g/ml). At the end of incubation, the nuclei were subjected to sequential digestion with RNase free-DNase and proteinase K; and labeled run-on RNAs (final volume 0.4 ml) were extracted as described in 'RNA extraction'. The final RNA pellet was washed with 70% ethanol, drained dry, resuspended in 200 μ l TES buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% SDS] and further diluted to 500 μ l with hybridization buffer. 5 μ l



aliquot of each nuclear RNA sample was tested for the incorporated radioactivity. Typically, total incorporated radioactivity ranged between $2-7 \times 10^6$ cpm/sample. However, the incorporated radioactivity/sample ranged only between $0.3-0.9 \times 10^6$ c.p.m. in the presence of α -amanitin.

Hybridizations cDNA strips were prepared in advance by slot-blotting excised rat PRL cDNA insert and linearized pBluescript (SK-) vector on a Nytran membrane. Each slot received 2 μ g of denatured DNA. The cDNAs on membrane strips were immobilized as described before (Blum, 1989); and prehybridized for 2 h at 55°C prior to the addition of run-on RNA samples.

Run-on RNA samples (with equivalent cpm in each sample) were hybridized with prehybridized cDNA strips at 55°C for 72 h. The strips were then washed sequentially as follows: (1) twice in $2 \times$ SSC for 1 h at 65°C; (2) twice in $0.1 \times$ SSC for 15 min at 65°C; (3) with 1 mg per ml RNase-A in $2 \times$ SSC for 30 min at 37°C. After the final wash in $2 \times$ SSC at 37°C for 1 h, the filters were blotted dry and autoradiographed.

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Quantification of autoradiograms

Autoradiograms were quantified by laser scanning densitometry using Pharmacia-LKB densitometer. Several film exposures were obtained to ensure that intensities of the images were within the linear range of the film.

Statistics

Where appropriate, the data were analysed by one way ANOVA and t-tests, and the level of significance was derived from a Newman-keuls test or a two-tailed table respectively.

Acknowledgements

I thank Drs R. Maurer and Dr J. Calvet for pPRL-1 and β -actin cDNA clones and Mr Benjamin J. Frey for expert technical assistance. This research was supported by a grant from National Institutes of Health, USA (DK-45044).

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